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A Rapid, Reliable, and Inexpensive Method for Detection of Di- and Trinucleotide Repeat Markers and Disease Loci From Dried Blood Spots

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We used a rapid and inexpensive method for studying the FMR1 CGG-repeat from dried blood spots, prepared from heel pricks, finger pricks, or an aliquot of blood from a venipuncture. The procedure includes a single tube for preparation of template DNA for PCR and minimal handling, avoiding opportunities for mislabelling specimens and loss of template. We extended the protocol to numerous di- and trinucleotide repeat markers and disease loci, including FRAXE, FRAXF, DXS548, DRPLA, and ZFY. The use of a highly reliable and very inexpensive method which employs blood spots as a source for target DNA means that newborn Guthrie cards can be used to establish allele frequencies for linkage disequilibrium studies, that large populations can be screened for genetic disorders, and that mapping studies can proceed rapidly even when only small amounts of blood are available from key family members. © 1996 Wiley-Liss, Inc.

KEY WORDS: trinucleotide and dinucleotide repeats, Guthrie spots, fragile X syndrome

INTRODUCTION

The fragile X syndrome, the most common inherited cause of developmental disability, is characterized by mild-to-severe cognitive impairment, behavior problems ranging from hyperactivity to autistic-like, and specific clinical findings in affected males. As the most common inherited cause of developmental disability, with estimates of incidence based on early chromosome testing ranging from 1/1,000–1/5,500 , there has been considerable discussion about population screening. With the identification of expansion of a CGG-repeat in the fragile X mental retardation-1 (FMR1) gene as the cause of fragile X syndrome, a means for accurate diagnosis and carrier assessment was developed, and recent testing of 10,000 females suggests that carrier incidence may be at least 1/259 [Rousseau et al., 1995]. Because of the high burden associated with this syndrome, and the need for sufficient counselling, educational, and other support services for newly-diagnosed families, we felt it was important to have accurate information on the incidence of both affected individuals and carriers in the general population. Further, we wished to test different models proposed for the generation of variable numbers of repeats using empiric data.

To achieve these goals, we studied the FMR1 repeat from Guthrie cards from 35,000 consecutive (anonymous) newborn males, and completed a pilot project of 2,050 males. For such a large study, it was necessary to adopt a rapid, reliable, and inexpensive method to amplify the FMR1 CGG-repeat region from the small amounts of blood available on newborn Guthrie cards. We also felt that the fewer the manipulations needed, the fewer the opportunities for labelling errors. Several methods have been reported for the extraction or elution of DNA from dried blood spots [McCabe et al., 1987; Rubin et al., 1989; Jinks et al., 1989; Cassol et al., 1991; Yourno and Conroy, 1992], but these generally involve multiple manipulations and do not always yield a template suitable for amplification of CG-rich sequences. The method we followed is so reliable and inexpensive, that we now routinely do all initial PCR studies from drops of blood, taken either by finger prick or aliquoted from a venipuncture sample, and we extended this protocol to the study of other tri- and dinucleotide repeat loci. Details of our methods and results on the following loci are reported here: the FMR1 CGG-repeat, which is expanded in individuals who are carriers or are affected with fragile X syndrome; the FRAXE GCC- and FRAXF $(GCCGTC)_n(GCC)_n$-repeats, which are expanded in individuals showing chromosomal fragility at the respective fragile sites; DXS548, a microsatellite locus located near the FMR1 CGG-repeat which is used for studies on

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founder chromosomes in fragile X syndrome; the DRPLA (dentato-rubral pallidoluysian atrophy) CAG-repeat, which is expanded in persons with this neurodegenerative disorder; and ZFY, a Y-chromosomal locus.

MATERIALS AND METHODS

Blood Spots

Blood spots used in the study were from three sources: newborn heel pricks (Guthrie cards), venipuncture samples submitted for routine testing, and finger pricks.

Newborn Guthrie cards. Newborn Guthrie cards with blood spots used for phenylketonuria (PKU) testing were obtained from the Ontario Ministry of Health, following ethics approval by the Research Ethics Board at Queen's University. In Canada, Guthrie cards are autoclaved prior to PKU testing.

Venipuncture samples. Upon arrival in the laboratory, a small amount of blood from all blood samples submitted for DNA testing was distributed onto filter paper using a Pasteur pipette. Sigma blood collection cards (Sigma Diagnostics Canada, Mississauga, Ontario) are convenient, but Whatman 3MM filter paper (Fisher Scientific, Nepean, Ontario) also works well.

Blood spots were also made using blood previously frozen at −70°C for up to 15 years in order to verify the "blood spot" method for amplification of the FMR1 CGG-repeat region from fragile X syndrome family members, including individuals with premutations and full mutations, as well as noncarriers.

Finger pricks. When it was not possible to do venipunctures, a small sample of blood was taken by finger prick. To ensure sufficient blood flow from the finger pricks, the hands and arm were washed with warm water for 1–2 min, followed by stroking the arm and hand downwards for a similar length of time. The hand was held downwards, the pulpy part of the finger was pricked with a sterile disposable B-D minilancet (Fisher Scientific, Nepean, Ontario), and the blood was placed on the filter paper. It is important that the collection card is well-soaked with the blood sample. Three or four spots of blood were taken per person.

Sample Preparation

Prior to use, all blood sample cards were autoclaved for 3–5 min [Carducci et al., 1992] and stored dry at room temperature until use. A clean 5-mm hole punch was used to remove a single spot (occasionally, two hole punches were used if the blood spot was very thin), which was placed into a sample tube with 100 μl of 1 × PCR buffer. Samples were boiled for 30 min and either used directly or stored at −20°C until use. *Immediately* prior to removing an aliquot for PCR, the sample tubes were again boiled for 10–20 min. One–3 μl of supernatant were used as the template for PCR reactions. Samples are stable in PCR buffer at −20°C for at least 20 months.

PCR Conditions

In general, PCR conditions were the same as those used for purified DNA preparations. Five, or rarely 10, extra cycles were added when blood spots were the source of template. A Perkin-Elmer 480 thermal cycler (Perkin Elmer-Applied Biosystems Inc., Mississauga, Ontario) or a Techne pHC-2 thermal cycler (Mandel Scientific, Guelph, Ontario) were used throughout. All samples were overlaid with mineral oil and denatured prior to temperature cycling. PCR products were mixed with formamide-loading buffer, heated for 5 min at 95°C, and separated by electrophoresis through a 4% or 6% denaturing polyacrylamide gel. Alleles were visualized by exposure to Kodak XAR-5 film.

FMR1 CGG-repeat. Primers used were those described by Fu et al. [1991]. Amplification was carried out using 3 μl of template in a 10-μl reaction containing 50 mM KCl, 2.2 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3, 200 μM dATP, 200 μM dTTP, 200 μM dCTP, 50 μM dGTP, 150 mM 7-deaza-dGTP, 2–3 μCi (alpha-^{32})dCTP, 10% DMSO, 0.5 U Taq polymerase (BRL), and 2 pmoles of each of the primers. Samples were denatured at 94°C for 5 min followed by 30 cycles (for Guthrie/blood spots) of 94°C, 1.5 min; 65°C, 1 min; 72°C, 2 min; and 72°C, 7 min.

FRAXE GCC-repeat. PCR conditions were as described by Knight et al. [1993], except that Taq polymerase and buffer were used, primer amounts were increased to 5 pmoles, and the number of cycles was increased to 40. Products were electrophoresed through a 4% denaturing gel.

DXS548, DRPLA CAG-repeat, and ZFY locus. The primers and PCR conditions used were those described by Snow et al. [1994] for DXS548, by Koide et al. [1994] for DRPLA, and by Witt and Erickson [1989] for ZFY, except that Taq polymerase was used, the number of cycles was 35, and 10 pmoles of each primer were used for ZFY.

RESULTS

We used the above-described protocol to analyze several triplet repeat and numerous dinucleotide repeat loci, and the results of some of these studies are shown here. Successful amplification was obtained for each of the loci attempted, and results were highly reproducible. In all cases, comparisons of PCR products from blood spots and purified DNA from the same individuals were made, and the sizes of the amplified regions were identical (data not presented). The most extensive comparison was of FMR1 PCR products from fragile X family members, including premutation and full mutation carriers. In addition, all samples referred for FMR1 testing which gave a repeat number of 35 or greater at the FMR1 locus were retested using a new hole punch, as well as using purified DNA if available. In all of these comparisons, identical FMR1 CGG-repeat numbers were obtained for blood spots and extracted DNA for all normal and premutation alleles of less than about 75 repeats. Larger FMR1 repeats were not detected using blood spots.

FMR1 CGG-repeat. Figure 1 shows a section of an X-ray illustrating the reliability of amplifying the FMR1 CGG-repeat from newborn Guthrie cards. The gel was double-loaded, and two rows of alleles are seen. A single sample in the bottom row did not amplify on this initial attempt, but did amplify in a repeat test. Blood spots from newborns do vary considerably with respect to the amount of blood present, and two spots

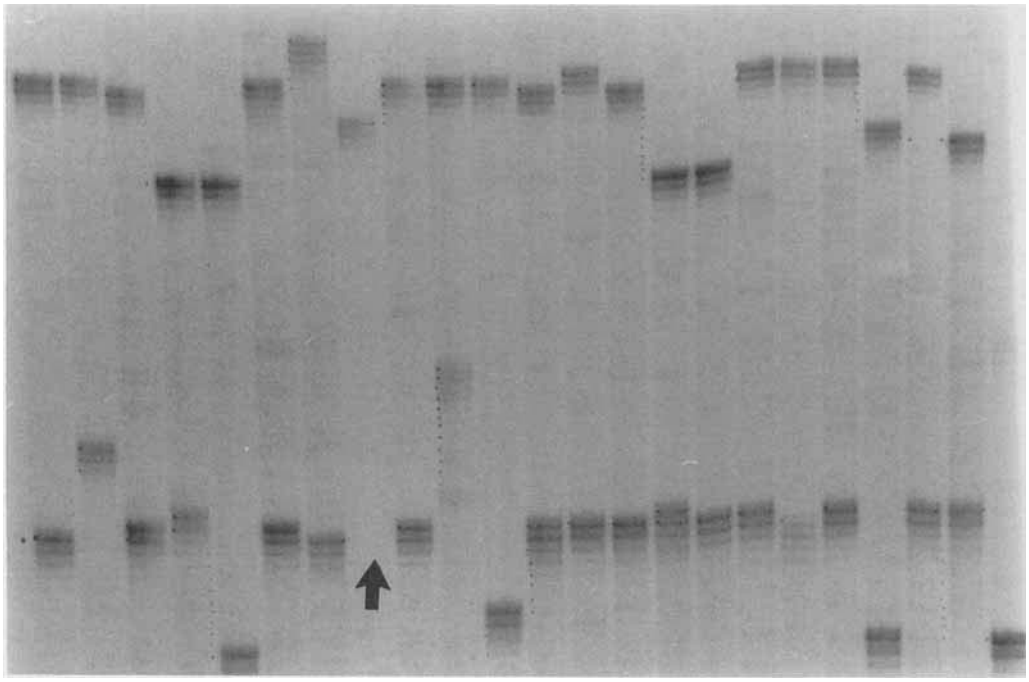

Fig. 1. Section of an X-ray illustrating the reliability of amplifying FMR1 CGG-repeat newborn Guthrie spots. Two rows of alleles are seen, reflecting double-loading of the gel. A single sample (arrow) did not amplify in this initial attempt.

may be required from some samples. Weaker signals seem to be correlated with the amount of blood present in the original blood spots, although this was not quantified. Of 2,050 consecutive newborn male blood spots tested, 1% required a second amplification, and all amplified (Holden et al., in preparation). The current cost per reaction for supplies is $1.25 (Canadian).

FRAXE GCC-repeat and FRAXF (GCCGTC)$_n$ (GCC)$_n$-repeat. Figure 2 shows examples of PCR amplification of these triplet-repeat expansion loci from blood spots and Guthrie cards, respectively. Again, the procedure for FRAXF (Fig. 2A) is highly reliable, with only one Guthrie card from 500 consecutive newborn males not giving a PCR product. Amplification of the

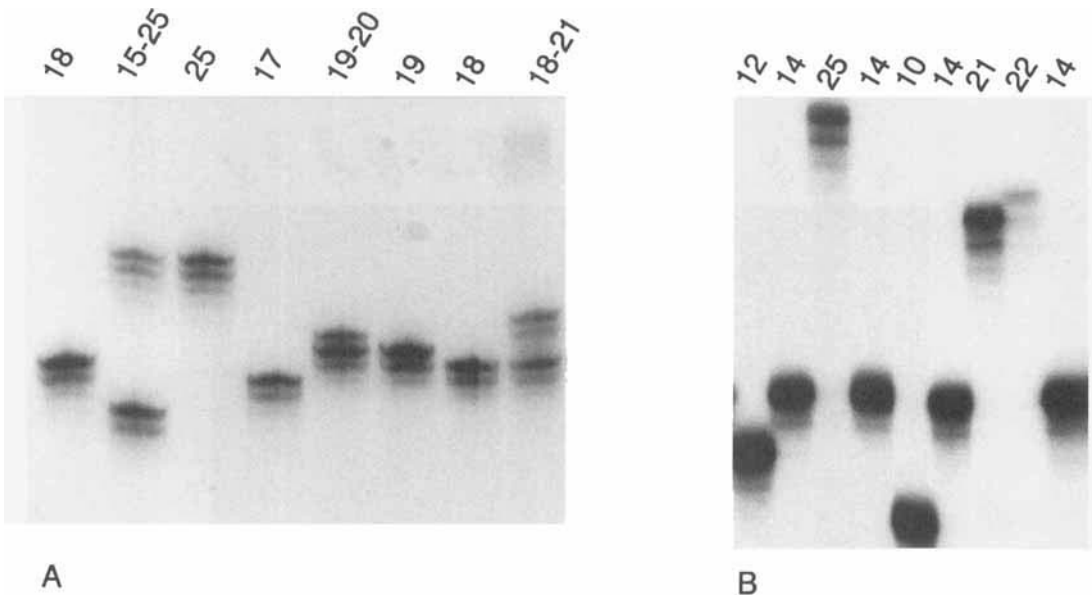

Fig. 2. Amplification of FRAXE GCC-repeat **(A)** and FRAXF $(GCCGTC)_n(GCC)_n$-repeat **(B)** from blood spots and newborn Guthrie spots, respectively.

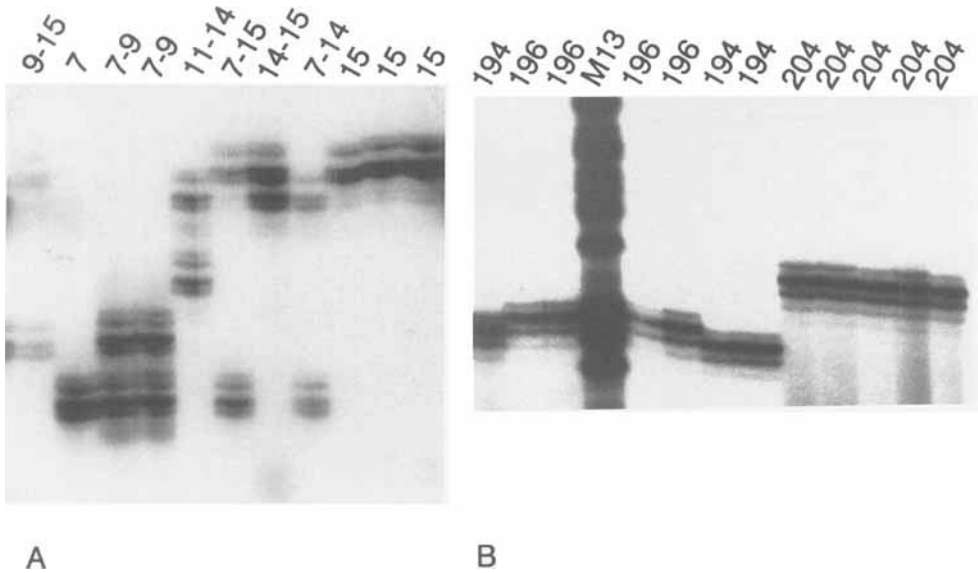

Fig. 3. Amplification of DRPLA CAG-repeat **(A)** and DXS548 **(B)** from newborn Guthrie cards.

GCC-repeat region in the FRAXE locus (Fig. 2B) is the only troublesome region we encountered in more than 30 loci tested using newborn Guthrie cards.

DRPLA CAG-repeat and DXS548. These loci are reliably amplified from newborn Guthrie cards (Fig. 3) and blood spots. Several examples of triplet repeat alleles at the DRPLA locus are shown in Figure 3A. Both alleles in heterozygotes are equally intense at the DRPLA locus. This is in contrast to findings at the FMR1 and FRAXE loci, in which the smaller allele in females is preferentially amplified during the PCR reaction.

Y-chromosomal sequences. Our population study examining the number of triplet repeats at the FMR1 and FRAXF loci involved testing newborn Guthrie cards from males only, since this avoided the problem of distinguishing between females who were homozygous for a specific allele and those who were heterozygous for a large (premutation or full mutation) allele which we might not detect. Some samples showed two distinct FMR1 or FRAXF alleles. To determine whether these samples were from XXY males or XX samples (either XX males or mislabelled female samples), we tested for the presence of Y-chromosomal sequences. Three loci were tested and all were found to amplify from at least some of the samples, as well as from control male blood spots and DNA, but not from control female blood spots and DNA. Figure 4 shows an example of amplification of the ZF locus on the Y-chromosome. Four of 10 blood spots were positive, indicating the presence of Y-chromosome sequences. Thus, it is possible to amplify sufficient DNA from Guthrie cards to yield ethidium-bromide visible bands.

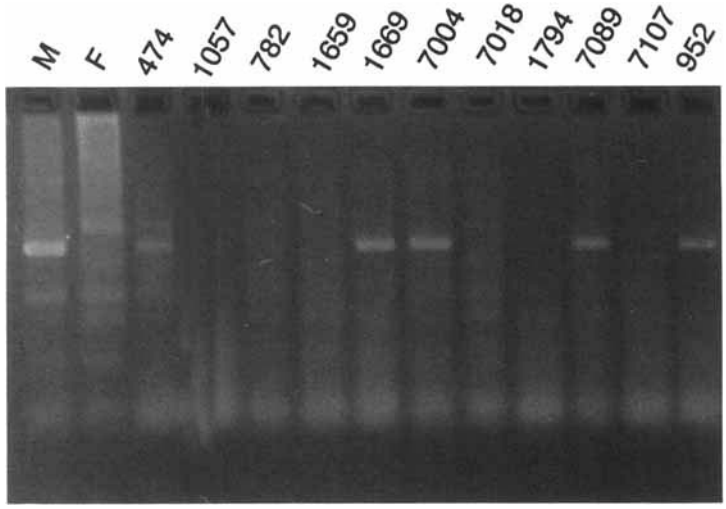

Fig. 4. Amplification of ZFY from Guthrie cards.

AGG-interspersion in the FMR1 CGG-repeat. It has been known for some time that the FMR1 CGG-repeat is cryptic, with interrupting AGGs [Verkerk et al., 1991]. It has been suggested that such interruptions serve as an anchor, preventing DNA slippage during replication [Levinson and Gutman, 1987]. We adapted the method described by Eichler et al. [1994] to detect interrupting AGGs within the CGG-repeat in some of the blood spots having intermediate or "grey zone" alleles. Figure 5 shows the FMR1 CGG-repeat configurations for seven blood spots tested in this manner.

DISCUSSION

A simple method for elution of template from blood spots for use in PCR reactions, which includes only pretreatment of the dry spots by autoclaving followed by boiling in PCR buffer, has been used successfully to amplify many di- and trinucleotide repeat markers and disease loci, including repeats with high CG content.

There are many applications for blood spots or newborn Guthrie cards as a source of DNA for PCR analyses. Our original intention was to use the newborn Guthrie cards for population studies on the FMR1 CGG-repeat. We extended that to other disease (e.g., FRAXE) and fragile-site (FRAXF, FRA16A, and FRA11B) loci and candidate genes for psychiatric disorders. Since neonatal screening for PKU is undertaken in most developed countries, these samples can be used to rapidly establish allele frequencies in the population for any locus being studied in relation to a specific genetic disorder. Using samples from individuals about whom there is no clinical or identifying information (and about whom only the sex or ethnic group is known) means that one is truly doing a population study, and not assuming that all "control" individuals are unaffected.

Introducing the flexibility that comes from doing assays on a blood spot, rather than on purified DNA, allows samples for DNA testing to be collected by persons not trained in venipuncture, since finger pricks can be done after only a few practice sessions. Transport of samples is easier and less expensive, since these can be packaged in biohazard bags and sent through regular post. Children who are afraid of needles are often less reluctant to provide a blood sample by finger prick. In cases where linkage studies have to be done in a hurry for someone who is pregnant and who has a family history of, for example, X-linked mental retardation, samples can be transported rapidly and there is no need for the time-consuming task of DNA extraction. The ability to store samples as dry blood spots on cards saves freezer space and means that one can collect samples from a large number of individuals with a particular clinical presentation or syndrome and undertake analysis of candidate genes up to several years later when the genes have been identified.

Finally, there is the increasing demand and interest in DNA testing for genetic disorders by the public. At current costs of reagents and supplies, it is essential that less expensive protocols be used whenever possible. The cost of supplies for most of the PCR reactions described here is in the $1.15–$1.50 range (Canadian). The simple boiling of a sample to obtain the appropriate template for PCR analysis means fewer reagents

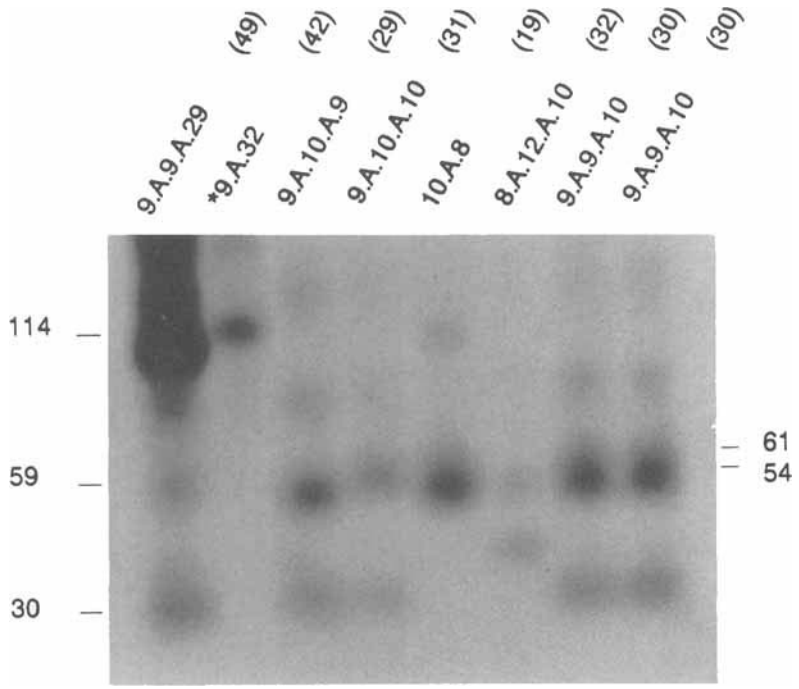

Fig. 5. Determination of AGG interspersions in FMR1 CGG-repeat using *MnlI* digestion of PCR products. Configurations for repeats and number of CGG repeats are indicated above each lane.

and less technical time per sample, and consequently more tests can be done for the same amount of money. Persons who might not otherwise have been able to afford such testing will have access to it.

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